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Methodological issues in assessing plasma 25-hydroxyvitamin D concentration in newborn infants☆,☆☆,★

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ABSTRACT

Background: Although no gold standard exists, liquid chromatography tandem mass spectrometry (LC–MS/MS) is a precise and accurate method for the analysis of plasma 25-hydroxyvitamin D (25(OH)D). Immunoassays are more readily available and require small volume sampling, ideal for infant testing. The objective was to compare two commercially available immunoassays for measuring circulating 25(OH)D concentration in infant plasma against LC–MS/MS.

Methods: Capillary blood samples from 103 infants were analyzed for plasma 25(OH)D using an enzyme immunoassay (EIA, Octeia, IDS Ltd.) and radioimmunoassay (RIA, DiaSorin). Plasma 25(OH)D_3, C-3 epimer of 25(OH)D_3 (3-epi-25(OH)D_3) and 24,25-dihydroxyvitamin D (24,25$(OH)_2D_3$) were measured on the same samples using LC–MS/MS. To establish whether plasma 24,25$(OH)_2D_3$ or 3-epi-25(OH)D_3 interferes with these immunoassay results, the zero 25(OH)D calibrator from each assay kit was spiked with increasing amounts of 24,25$(OH)_2D_3$ or 3-epi-25(OH)D_3.

Results: Classifying infants below the common vitamin D status targets of 50 nmol/L and 75 nmol/L respectively, 58% and 99% fell below using the RIA, 19% and 56% with the EIA and 31% and 76% with LC–MS/MS. Compared to LC–MS/MS, both immunoassays showed poor Bland–Altman limits of agreement for 25(OH)D concentrations (RIA: limits of agreement −27 to +13%; EIA: −12 to +41%), and mountain plots (folded cumulative distribution) depicted significant skew and bias. Spiked 24,25$(OH)_2D_3$ concentrations, but not 3-epi-25(OH)D_3, appeared as >100% of known values on the EIA but not on the RIA thus, suggesting that the EIA may cross-react with 24,25$(OH)_2D_3$ to a greater extent than 3-epi-25(OH)D_3.

Conclusion: Two common immunoassays resulted in very different classifications of vitamin D status possibly related to the interference of other vitamin D metabolites. Based on these data, LC–MS/MS assessment of vitamin D status is recommended in young infants (4–6 weeks of age).

Introduction

Circulating 25-hydroxyvitamin D (25(OH)D) is the best indicator of vitamin D status [1]. Blood concentrations of 25(OH)D <30 nmol/L are generally consistent with deficiency [1]; however, the concentration consistent with positive health outcomes is an ongoing area of controversy. The Canadian Paediatric Society (CPS) denotes serum 25(OH)D between 75 and 225 nmol/L as the target for vitamin D status [2] yet the Institute of Medicine and the American Academy of Pediatrics recognize the 50 nmol/L target to support bone health [1,3].

A number of assays are now available for measurement of 25(OH)D in serum or plasma. Measurements made using high performance liquid chromatography (HPLC) with UV detection have long been accepted as the official method of standards (e.g., U.S. Pharmacopeial Convention,

Abbreviations: EIA, enzyme immunoassay; RIA, radioimmunoassay; 25(OH)D, 25-hydroxyvitamin D; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 1,25$(OH)_2$D, calcitriol; HPLC, high performance liquid chromatography; LC–MS/MS, liquid chromatography tandem mass spectrometry; IDS, Immunodiagnostic Systems Limited; DEQAS, Vitamin D External Quality Assessment Scheme.

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European Pharmacopoeia, Association of Analytical Communities) for measuring vitamin D. Recently liquid chromatography tandem mass spectrometry (LC–MS/MS) systems have been used for more rapid, specific and sensitive assessment [4] and is gaining wide-spread acceptance [5–7]. Immunoassays continue to be commonly used in the clinical setting for the assessment of 25(OH)D concentration due to their ease of use. Although, interpretation of assay results is further complicated by proprietary antibodies having different affinities for vitamin D metabolites or epimers, such as 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) or C-3 epimer of 25(OH)D (3-epi-$25(OH)D_3$). Plasma concentrations of $24,25(OH)_2D$ have been reported to be low (<10 nmol/L) and represent <6% of circulating 25(OH)D [8]; however, 3-epi-$25(OH)D_3$ has been detected in 20–98% of infants using LC–MS/MS [6,9] and may represent up to 40% of total plasma 25(OH)D measured in young infants [6,10].

Due to the ongoing debate as to what threshold of vitamin D status best supports bone health in children, establishing accurate assessment methods is key. Despite the limitations of commercial immunoassays, the low specimen volumes required are critical for allowing measurement of vitamin D status in young infants and additionally not all laboratories have access to HPLC with UV detection or LC–MS/MS technology. Specifically, the DiaSorin-RIA has the capacity to equally detect the $25(OH)D_2$ and $25(OH)D_3$ isoforms arriving at total 25(OH)D in 50 μL of plasma sample. Similarly, Immunodiagnostic Systems Limited (IDS) has developed a low sample volume (25 μL) enzyme immunoassay (EIA: Octeia) which performed similarly to the DiaSorin-RIA in the recovery of $25(OH)D_3$ from spiked adult samples [11]. Both assays are specific for $25(OH)D_3$; however, the EIA appears to underestimate $25(OH)D_2$ (specificity of 75%) [12]. Moreover, neither of these assays has been validated to assess infant vitamin D status or to explore prevalent isoforms/metabolites during infancy [6].

The main objective of this study was to compare two commercially available assays (DiaSorin-RIA and IDS-EIA) to a LC-MS/MS method (selected here as the 'nominal' gold standard) for measuring plasma 25(OH)D concentrations in healthy infants. Secondly, we sought to establish whether metabolites of vitamin D, such as $24,25(OH)_2D$ and 3-epi-25(OH)D, might be detected by these immunoassays and potentially interfere with the assessment of plasma 25(OH)D.

Materials and methods

Newborns were recruited from pediatric clinics located in the greater Montréal area, as part of a prospective trial of vitamin D supplementation in breastfed infants up to 1 year of age, from March 2007 to August 2010 [9]. The present study only included baseline data from infants 4–6 weeks of age, who may have already started vitamin D_3 supplementation of 400 IU/day prior to recruitment. Infants were eligible for the study if they were healthy, singleton, term infants born of appropriate size for gestational age, and to healthy breastfeeding women (consuming >80% of feeds from breast milk). Exclusion criteria included infants of mothers with a history of gestational diabetes or hypertension in pregnancy, chronic alcohol use and malabsorption syndromes (celiac and Crohn's disease). The main study and secondary analyses were approved by the Institutional Review Board (IRB) of McGill University (A05-M61-06A/A06-M71-13A). Parents gave written informed consent.

Study visits took place at the Mary Emily Clinical Nutrition Research Unit of McGill University. Anthropometric measurements were taken and included weight, length and head circumference. Birth anthropometry, gestational age and vitamin D supplementation since birth were reported by the mother. Capillary blood samples, collected by heel lance in tubes containing sodium heparin, were centrifuged (2235 ×g for 20 min at 4 °C) and stored frozen at −80 °C until batch analysis.

Samples were measured for total plasma 25(OH)D using both EIA (Octeia, IDS Ltd., Boldon, UK) and RIA (DiaSorin, Stillwater, MN, USA). The IDS-EIA uses a polyclonal sheep-antibody-coated microplate and a biotinylated 25(OH)D tracer with a proprietary buffer reagent for dissociating 25(OH)D from its binding proteins. In contrast, the DiaSorin-RIA uses a goat antibody and a ^{125}I-labeled 25(OH)D tracer with an immunoprecipitation step. Both report precision <12.5% and sensitivity of 6 nmol/L (2.4 ng/mL).

Although, no clear gold standard method currently exists for the assessment for circulating 25(OH)D concentrations, chromatographic methods allow the assessment of vitamin D epimers and metabolites. Plasma $25(OH)D_3$, 3-epi-$25(OH)D_3$ and $24,25(OH)_2D_3$ were quantified by Warnex Bioanalytical Services (Laval, Québec) using a sensitive LC–MS/MS method after Diels–Alder derivatization as described in [9]. As expected, all infants had vitamin D_2 metabolites below the limit of quantification, as none were receiving vitamin D_2 supplements. The vitamin D status of infants was categorized (using LC–MS/MS) as plasma 25(OH)D ≤29.9 nmol/L (deficient), 30–49.9 nmol/L, 50–74.9 nmol/L and ≥75 nmol/L [2]. The ≥50 nmol/L cut-off was included as a target as defined by the American Academy of Pediatrics [3] and the ≥75 nmol/L cut-off is recognized by the Canadian Paediatrics Society and others [2,13]. The intra-assay coefficient of variation was less than 15% for all vitamin D metabolites across all assays (Supplemental Table 1) including assay kit controls from the manufacturer which met the specifications. Both laboratories participate in the DEQAS (Vitamin D External Quality Assessment Scheme) program and obtained Certificates of Proficiency, which reflect that 80% or more of the reported results fell within 30% of the ALTM (All-Laboratory Trimmed Mean). Since 2011 the National Institutes for Standards and Technology (NIST) has instituted chromatographic techniques for their certified reference values for different levels of vitamin D metabolites [14]. At the time of the study measures the NIST vitamin D controls were not available; however, these were tested subsequently for our assay and found to be accurate within 7% of the certified concentrations.

The manufacturer's specifications for both 25(OH)D assays were similar for analytical specificity according to the percent cross-reactivity with different vitamin D metabolites. The DiaSorin-RIA is reported to cross-react with the $24,25(OH)_2D$ metabolite at 100% and <1% with 3-epi-25(OH)D. The cross-reactivity of the IDS-EIA was reported as >100% for $24,25(OH)_2D$ but, was not reported for 3-epi-25(OH)D. To establish whether these analytes could interfere with 25(OH)D results, the zero calibrator from each 25(OH)D kit was spiked with increasing amounts of $24,25(OH)_2D_3$ (Enzo Life Sciences cat # DM-300) and 3-epi-$25(OH)D_3$ (Sigma Aldrich, cat # 705993) standards. Standards were dissolved in 100% ethanol [11] to a concentration of 125 μmol/L and this stock solution was added to zero calibrator (cal 0) to obtain dilutions of $24,25(OH)_2D_3$ at 0, 9.6, 19, 29, 38, 58, 77, 115 and 154 nmol/L and 3-epi-$25(OH)D_3$ at 0, 9.6, 38, 77, 115 and 154 nmol/L. Absorbance readings were interpolated using the 25(OH)D standard curve to estimate cross-reactivity. All $24,25(OH)_2D_3$ and 3-epi-$25(OH)D_3$ standards were measured on both assays in duplicate or triplicate on ≥2 occasions using kits with different lot numbers. All immunoassays were run in accordance with manufacturer's specifications. In order to establish that standards reacted similar to human samples, pooled cord plasma samples of 4 term infants with plasma 25(OH)D measurements of <20 nmol/L measured by EIA, were also spiked with increasing concentrations of $24,25(OH)_2D_3$ and 3-epi-$25(OH)D_3$, using the same dilutions as with the zero calibrator, and tested on both assays in duplicate or triplicate.

Statistical analysis

All descriptive characteristics for infants are expressed as arithmetic mean (± standard deviation). To further describe the population, infants were also categorized by plasma 25(OH)D concentration. Mean differences in plasma 25(OH)D concentration among methods were evaluated using a Kruskal–Wallis test with Dunn's test for multiple comparisons. The relationship between immunoassays was described using a quadratic–plateau model as assessed by segmental polynomial (knot) regression (SAS PROC NLIN; Example 60.1 Segmented Model)